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PRINCIPAL INVESTIGATOR: Saraswati Sukumar, Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins University School of Medicine
Baltimore, Maryland 21205-2196

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Saraswati Sukumar, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205-2196

E-Mail: saras@jhmi.eud

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We proposed to test the validity of the hypothesis that introduction of recombinant toxins into the confines of the mammary ductal tree through the teat will kill breast epithelial cells. To this end, we designed a new toxin to target epithelial cells. We have completed the construction of a chimeric toxin consisting of the protein transduction domain of the HIV TAT gene to target and enter the cells, and the VPR gene of HIV to cause apoptosis. Expression of this protein in bacteria, and its purification is in progress. In animal experiments, we also demonstrated the concept that both chemopreventive and chemotherapeutic drugs could be clinically effective when delivered by the intraductal route. This allows us to ask if, by this route of administration, systemic toxicity could be avoided. In the third year of this grant we will test the efficacy of this toxin in cultured human and rat breast cells. If effective, we will test the toxin in the rat model system.

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INTRODUCTION

Women with a strong family history of breast cancer may have up to an 80% risk of developing breast cancer over their lifetime. Given the rising risk, and the increasingly identifiable high-risk group, the time has come to give serious consideration to the options available to prevent breast cancer. The matter has acquired a sense of urgency in the last three years because of two seminal discoveries in the genetics of hereditary breast cancer. Individuals with a family history of breast cancer (comprising 5-10% total breast cancer cases) often carry a mutation in the breast cancer susceptibility genes BRCA1, BRCA2 or p53 and ATM are at particularly high risk of developing breast cancer at a young age. As more women test positive for mutations in BRCA1 and BRCA2, the question of how best to manage these patients becomes ever more pressing. Unless reliable and effective methods for preventing breast cancer can be devised, determining susceptibility to breast cancer may be useless and possibly even psychologically detrimental. As more breast cancer associated genes are identified, particularly among the larger population of women without a strong family history, preventive strategies with minimal side effects are clearly needed.

BODY:

This proposal seeks to test the radically new idea that breast cancer prevention can be achieved by selectively killing the cells that line the ducts from which the majority of malignant breast cancers arise. Multiple strategies could be applied to selectively kill breast epithelial cells. One method is to use proteins called ligand/toxin conjugates. Ligand/toxin conjugates combine their cytotoxic properties with the ability to selectively target cells carrying specific growth factor receptors. Cells that do not express the receptors remain unaffected. However, our work in the first year showed that, unlike their effects on human cells, TGFalpha-Pseudomonas exotoxin had no effect on rat mammary glands even when administered in milligram quantities. In view of the ineffectiveness of the exotoxin conjugates to kill rat epithelial cells, we designed and began to synthesize a new toxin for possible use in the intraductal ablation of mammary epithelial cells.

The revised objectives of our grant included:

1. Construct the TAT-VPR fusion protein.
2. Synthesize it in amounts sufficient to perform in vitro toxicity in tissue culture and in vivo studies in the rat carcinogen-induced mammary tumor model.
3. In addition to prevention, establish the utility of the intraductal approach to breast cancer therapy of rat tumors as a means of reducing toxic side effects that result from systemic treatment. If successful, test utility of fusion toxin to therapy of preneoplastic and later stage disease in the rat model.

Objective 1:
Construction of a TAT-VPR fusion protein:

The TAT component: Rather than use retroviral or lentiviral vectors for efficient transduction of the desired gene products, direct injection of proteins is gaining popularity because it avoids the possibility of low efficiency targeting, short term expression of the desired protein, and fear of recombination events that might render the viral vectors of some danger to the recipient. Direct delivery of the gene product would be ideal. Delivery of therapeutic compounds, peptidyl mimetics and proteins into cells is, however, limited by the size of the proteins, typically less than 600 daltons.

Recently, Steven Dowdy's group (1) devised a new way of delivering target proteins to cells. Full length fusion proteins were generated that contain the NH2-terminal 11 amino acid protein transduction

domain (PTD) from the human immunodeficiency virus (HIV) TAT protein. These proteins are prepared under denaturing conditions. Protein transduction is thought to occur lipid bilayer component of the cell membrane. Thus, in principle all mammalian cells should be susceptible to protein transduction. The authors have succeeded in using this technology to transduce over 50 proteins ranging in size from 15-120 kD into a wide variety of human and murine cell types in vitro (1).

Tagged with the 116 kD b-galactosidase or with a small oligomer tagged with fluorescein isothiocyanate (FITC), the proteins were visible all over the body, including the brain. Thus the small 11-amino-acid sequence from the TAT protein of HIV is sufficient to provide entry into all the cells of the body (1).

This methodology opened up new possibilities for the development of vaccines and protein therapies for cancer and infectious diseases. Potential immune responses and toxicity associated with long term transduction in vivo are important issues that remain to be examined. However, injection of a mouse with 1 mg of TAT PTD fusion protein/kg body weight each day for 14 consecutive days produced no sign of gross neurological problems or systemic distress.

The Viral Protein R (VPR) component:

HIV-1 VPR is a 96 amino acid protein that is expressed after HIV-1 infection. A number of functions have been ascribed to the VPR, including induction of G2 cell cycle arrest and apoptosis in T-cells and other human cells. VPR was more effective in rapidly dividing cells rather than in slow growing cells, and had an apoptotic effect on a number of cancer cell lines (2, 3), possibly by dissipation of mitochondrion membrane potential by inducing lipidic pores, thus causing release of cytochrome C and apoptosis inducing factor. In subsequent studies, the region of the VPR gene that conferred the apoptotic properties was identified as residing in 54 amino acids (from 42-96). Thus, the full length VPR protein was not essential- just the 54 amino acid stretch could cause apoptosis of rapidly dividing cells. The differential effects of the VPR protein fragment on rapidly dividing cells and most cancer cells versus normal (slow dividing) cells is not yet clear, and needs detailed further studies (2). However, it provides proof of concept for potential adaptation of the unique properties of VPR in the setting of cancer.

The TAT-VPR fusion protein: Combining the strength of the TAT protein's ability to enter all the cells- irrespective of whether they are dividing or not, and the ability of Vpr to induce G2 arrest and apoptosis in dividing cells, we designed a vector containing the following features. The protein contained the 11 amino acid TAT cell entry sequence, followed by 54 amino acids of the Vpr protein. A methionine was added at the 5'end, and a V5 and HIS tag are present at the 3'end. The resulting protein is small- approximately 10-13 kD in size.

Construction of the fusion protein: Both sense and antisense oligonucleotides corresponding to the 68 amino acids for the TAT-Vpr construct were synthesized, annealed, and PCR-amplified. The product was cloned into pUni/V5-His-TOPO vector (Echo cloning system from Invitrogen). The sequence was verified by nucleotide sequencing. The sequence was then recombined into an acceptor vector (E.coli expression vector) using the cre-lox site specific recombination system.

We confirmed in pilot experiments that this construct is expressed as a 10-13 kD protein in a cell free system. Recently, we have expressed it in E.coli (BL21codon T) after induction with IPTG. The protein was expressed in small quantities, and then failed completely. We are currently attempting to express the protein in a variety of modified E.coli. It is possible that the high proportion of asparagine and leucine in the construct render it difficult for E.coli to express this protein. We will seek the help of protein experts

to overcome this problem. Generating sufficient quantities of this protein will enable us to proceed with objective 2

Objective 3: To test the efficacy of the intraductal approach to prevention of breast cancer, we tested the effects of 4-hydroxy tamoxifen in rats. In the treatment setting, treatment was initiated when the tumors were 5 mm in size. 100 ul of 4-hydroxytamoxifen (5 ug/ul) was administered at weekly intervals by the intraductal route. A third group received 4 OH-tamoxifen subcutaneously at the same dose, the usual and proven effective way of administration.

In the prevention setting, administration of 4OH-Tmx was initiated (same dosage as above) two days after administration of the carcinogen, and continued for 4 months, the time after which tumors arose in control untreated rats. In the treatment setting, treatment was initiated when the tumors were 5 mm in size, and continued at weekly intervals for four months thereafter. A third group received 4 OH-tamoxifen subcutaneously, the usual and proven effective way of administration. The rats were euthanized at when their tumors reached 15 mm in size. Tumor free rats were observed for 12 months.

Group	No. of rats	No. of tumors
Prevention:		
NMU	12	20
NMU+4 OH-Tmx.i.d	12	2
NMU+ 4OH-Tmx, s.c.	12	0
Treatment:		
NMU alone	16	17
NMU+4OH-Tmx., i.d	15	1
NMU+ 4OH-Tmx, s.c.	10	0

The results of this experiment revealed that the intraductal route of injection of chemotherapy is effective using a well-known agent 4-hydroxy tamoxifen at doses comparable to that given subcutaneously in tumor bearing rats. Furthermore, the agent is promising in a prevention setting as well, where tumors arose at a low incidence and with a significantly later time of appearance. The therapy experiments will be conducted with new agents such as arsenic alone and in combination with taxol, and with doxorubicin. On completion of objective 2, experiments will be undertaken in animals using the new toxin.

KEY RESEARCH ACCOMPLISHMENTS:

-succeeded in constructing the fusion protein vector, and observed the expression of correct size protein transiently in E.coli.

-performed studies to test the efficacy of the intraductal route of administration of a chemotherpaeutic/ chemopreventive compound, 4 OH-tamoxifen in animal experiments.

REPORTABLE OUTCOMES:

Presentations and publications:

The effects of 4 OH-tamoxifen in the rat mammary tumor model was presented as a poster at the American Association of Cancer Research meetings in 2000, and is printed as an abstract in its Proceedings.

CONCLUSIONS:

This project has been a challenging one in that the compound that was effective in humans for which the proposal was submitted had no effect on rodents. While it is highly toxic in humans, both rats and mice displayed no effects. So we changed our strategy and designed a new toxin, based upon newly emerging concepts. The synthesis of this toxin is proving quite difficult- and technical problems need to be overcome. On another front, we hypothesized that the intraductal approach may be useful for therapy as well, particularly in view of the recent developments in ductal lavage catheters that are able to access ducts with ease. We have seen very encouraging results here, and we will continue to test this concept using existing and novel drugs.

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APPENDICES: None